Purification of a troponin I-like factor from pig platelet

E. der Terrossian*, G. Pruliere and A. d'Albis

Laboratoire de Biologie Physicochimique, Bât. 433, Université Paris-Sud, 91405 Orsay, France

Received 15 November 1982

A troponin I-like factor has been purified from pig platelet by G150 Sephadex filtration of a low ionic strength extract, acidification at pH 4.2, ion exchange on DE-52 cellulose, and affinity chromatography on calmodulin-Sepharose. This protein (M_r 17000), together with pig brain calmodulin and platelet tropomyosin, is able to participate to the reconstitution in vitro of a thin filament-like complex which modulates with 55% calcium sensitivity** the platelet actin-activated Mg^{2+}-dependent ATPase activity of rabbit skeletal muscle myosin.

Pig platelet *Troponin I-like factor* *Tropomyosin* *Actin-linked regulation* *Calcium sensitivity* *Calmodulin*

1. INTRODUCTION

There is widespread agreement that platelet responses to stimulation involve a family of proteins similar to those implicated in the mechanism of muscular contraction. Regulation of platelet motility has been related, as in smooth muscles, to the state of phosphorylation of the 20000 M_r subunit of myosin, the phosphorylation being catalyzed by the complex formed in the presence of calcium between a specific light chain kinase and calmodulin (for reviews [1,2]). Besides this myosin-linked control, another type of calcium regulation involving the thin filament proteins as in striated muscles has also been suggested [1,3,4]. In addition to actin, this system would implicate the presence of both tropomyosin and troponin in platelets. Purification of platelet tropomyosin from various species has effectively been achieved [5–7] whereas that of troponin has not been described yet.

We have previously reported that highly purified pig platelet tropomyosin possesses regulatory properties which are similar to those of its skeletal muscle counterpart since it binds to platelet actin and skeletal muscle troponin to form a Ca^{2+} sensitive complex [8]. On the other hand, it has been observed that in the presence of tropomyosin isolated either from striated muscle [9] or from platelet ([10,11] and unpublished results) the complex formed between troponin I and calmodulin can be substituted to the whole troponin molecule in the assembly of a hybrid and fully active Ca^{2+}-sensitive thin filament-like system. Since platelets contain actin, tropomyosin and calmodulin, we have looked for the presence of a troponin I-like component in these cells.

In this study***, we describe the purification from pig platelets and the preliminary characterization of a 17000 M_r protein which binds to calmodulin in a Ca^{2+} dependent manner. Together with tropomyosin it leads to more than 50% inhibition of the platelet actin-activated Mg^{2+}-dependent ATPase of rabbit skeletal muscle myosin in the presence of 2 mM EGTA, full activity being restored by 0.2 mM Ca^{2+}.

* To whom correspondence should be addressed

** Calcium sensitivity = [1 − (ATPase EGTA/ATPase calcium)] × 100

*** Part of this work has been presented at the 11th European Congress on Muscle and Motility (Leicester, 1982)

Abbreviations: PAGE, polyacrylamide gel electrophoresis; TLCK, tosyl lysyl chloromethyl ketone; EGTA, ethylene glycol-bis (β-aminoethylether)-*N,N'*-tetraacetic acid; EDTA, ethylenediamine tetraacetic acid

Published by Elsevier Biomedical Press
00145793/83/0000–0000/$3.00

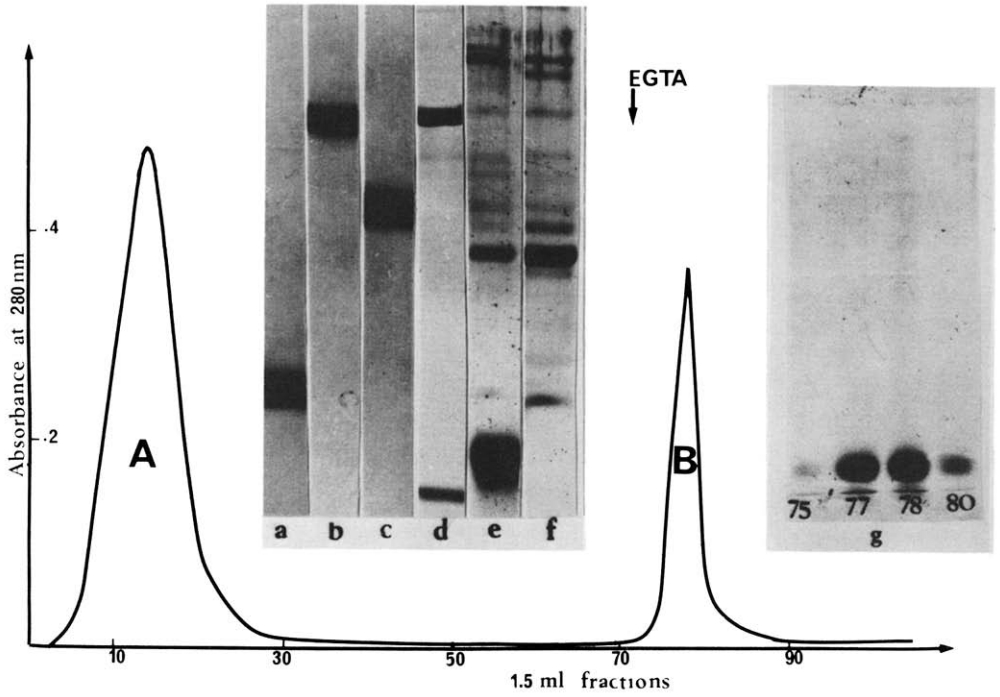

Fig.1. Affinity chromatography of the platelet inhibitory protein on calmodulin-Sepharose column containing 10 mg calmodulin bound as outlined by Klee [25]. *Insert:* SDS-PAGE of (a) calmodulin; (b) actin; (c) tropomyosin; (d) profilactin; (e) fraction loaded on calmodulin-Sepharose; (f) peak A; (g) fractions 75, 77, 78, 80 of peak B.

2. MATERIALS AND METHODS

2.1. *Preparation of proteins*

Ca^{2+} insensitive rabbit skeletal myosin, prepared according to [12], has been kindly provided by Dr J.-J. Béchet. Calmodulin was prepared from pig frozen brain as in [13]. Platelet tropomyosin was prepared as in [7] and platelet actin as will be described elsewhere. These proteins were more than 97% pure as judged by PAGE (fig.1). Protein concentrations were determined either spectrophotometrically with E 1%/290 nm = 6.5 for actin and E 1%/280 nm = 5.6 for myosin or by the colorimetric method of Bradford [14] using bovine serum albumin as a standard.

2.2. *Polyacrylamide gel electrophoresis*

PAGE was carried out either in SDS or in urea on 12.5% polyacrylamide slab gels as in [15]; staining was performed with fresh solutions of 0.2% Coomassie Brilliant Blue.

2.3. *ATPase assays*

The platelet actin-activated Mg^{2+}-dependent ATPase activities of rabbit skeletal muscle myosin were measured in 30 mM KCl, 30 mM imidazole (pH 7), 5 mM $MgCl_2$, 0.5 mM DTT, 1 mM NaN_3, in the presence of either 0.2 mM $CaCl_2$ or 2 mM EGTA. 1 ml of assay contained 100 μg rabbit skeletal muscle myosin, 200 μg platelet actin (M_r 42000), and depending on the assay 95 μg platelet tropomyosin (M_r 60000), 22 μg inhibitory protein (M_r 17000), 28 μg calmodulin (M_r 18000). The mixture was equilibrated under gentle stirring at 29°C for 10 min. The enzymatic reaction was then initiated by the addition of 2 mM [γ-^{32}P]ATP. 60 μl aliquots were removed at indicated times, and the γ-^{32}P released was measured by the method of Seals et al. [16]. The specific radioactivity of [γ-^{32}P]ATP checked for each assay was 2.10^6 cpm ± 4000/μmol ATP.

3. RESULTS AND DISCUSSION

Our approach to the characterization of the actin-linked control of motility in platelets has been to resynthesize in vitro, an active thin filament-like system with highly purified proteins isolated from the cells.

3.1. *Purification*

Our strategy for the purification of a troponin I-like component from platelets was based on the specific properties of the muscle protein: (1) it can be separated from tropomyosin by the isoelectric precipitation of this latter protein at pH 4.2 in high salt [17]; (2) it is dissociated from the other troponin subunits in 1 mM EDTA and does not bind to DE-52 cellulose in 6 M urea at pH 8 [18]; and (3) it interacts with calmodulin at physiological salt concentration in the presence of Ca^{2+}, but not in its absence [9,19].

3.1.1. Platelet extraction, gel filtration and tropomyosin isoelectric precipitation

Pig platelets were prepared and stored at −20°C in 40% glycerol as in [7]. Proteins extraction in 5 mM sodium phosphate (pH 7.5), 10 mM NaCl, 0.5 mM $CaCl_2$, 2 mM NaN_3, 1 mM PMSF, 0.25 M sucrose was followed by high-speed centrifugation and filtration of the supernatant on G150 Sephadex in 2 mM Tris–HCl (pH 7.5), 0.2 mM $CaCl_2$, 0.2 mM ATP, 0.5 mM DTT, 3 mM NaN_3. Two main fractions were obtained: the first one containing essentially high molecular weight proteins, F-actin and tropomyosin, the second one mainly G-actin used for the further purification of this protein as will be fully detailed elsewhere. Isoelectric precipitation of tropomyosin at pH 4.2, in 1 mM KCl was then achieved and the pellet collected at 40000 × *g* was used for further purification of tropomyosin as in [7]. The supernatant (130 ml) was readjusted to pH 7, concentrated to about 10 ml over sucrose, and then exhaustively dialysed versus 50 mM Tris–HCl (pH 8), 1 mM EDTA, 1 mM DTT, 3 mM NaN_3 (buffer I). Some denatured material was removed by filtration successively through 8 μm and 0.45 μm Millipore membranes.

3.1.2. Ion-exchange chromatography

The clear solution of proteins was made 6 M with solid urea and stirred gently for 30 min. It was then loaded on a (11 × 2) cm DE 52-cellulose column equilibrated overnight with buffer I containing 6 M urea. The void volume was concentrated to 5 ml over sucrose, exhaustively dialysed versus 1 M KCl, 25 mM Tris–HCl (pH 7.5), 1 mM $MgCl_2$, 1 mM $CaCl_2$, 0.5 mM DTT, 2 mM NaN_3, and finally versus this same buffer containing 150 mM instead of 1 M KCl (buffer II). The proteins which do not bind to the cellulose display three major bands on SDS-PAGE (fig.1), with app. M_r of about 50000, 25000 to 26000, and 17000 respectively. This last broad predominant band migrates between calmodulin (18000 M_r) and profilin (15500 to 16000 M_r). Some minor bands were also observed.

3.1.3. Affinity chromatography

Following dialysis, the flowthrough of DE-52 cellulose was submitted to affinity chromatography on a (0.9 × 30 cm) calmodulin–Sepharose column equilibrated with buffer II (fig.1). Peak A contains most of the UV-absorbing material which passed unimpeded through the column in 1 mM $CaCl_2$ and 150 mM KCl whereas peak B, eluted when 1 mM EGTA replaced 1 mM $CaCl_2$ in the same buffer, contains only the 17000 M_r component. The content of the two peaks was concentrated over sucrose and then dialysed versus 50 mM KCl, 50 mM imidazole (pH 7), 2 mM NaN_3, 0.5 mM DTT. After addition of sucrose at a final concentration of 5 mg/ml, the solutions were freeze-dried. Before use, the proteins were dissolved in distilled water and dialysed versus the desired buffer. The successive steps of the purification of the 17000 M_r protein are summarized in table 1. Total yield was between 0.5 mg and 1 mg per 4 g wet weight cells.

3.2. *Partial characterization*

The behaviour of the 17000 M_r protein on DE-52 cellulose at pH 8, and the fact that it does not migrate at all in 6 M urea PAGE at pH 8.6 suggest that it is a basic protein. It resembles one of the proteins described in the actin-linked regulation of uterine smooth muscle [20]. It is very labile, and proteolysis sometimes occurs during purification (fig.1) and after storage at −20°C with the formation of a polypeptide which appears at the front of the 12.5% polyacrylamide gel. To check

Table 1

Purification of a troponin I-like factor from pig platelet

Pig platelets stored in 40% glycerol; lysed in 10 volumes of Ca^{2+}, ATP, and sucrose buffer at pH 7.5; centrifuged at 100000 × *g* for 1 h

- Supernatant: filtrated on G150 Sephadex (These two first steps as in [7])
- Cellular debris (discard)

- High molecular mass proteins, F-actin, tropomyosin fraction made 1 M with KCl, adjusted to pH 4.2 and centrifuged at 40000 × *g*
- G-actin fraction (used for further purification of this protein)

- Supernatant: readjusted to pH 7, concentrated over sucrose, dialysed versus Tris–HCl (pH 8), 1 mM EDTA, made 6 M with urea and loaded on DE-52 cellulose
- Tropomyosin pellet (used for further purification of this protein) as in [7]

↓

Flowthrough

dialysed versus 1 mM Ca^{2+}, 150 mM KCl, 1 mM Mg^{2+}, 25 mM Tris–HCl (pH 7.5) loaded on calmodulin-Sepharose.

Eluted with 1 mM Ca^{2+} ⟶ peak A.

Eluted with 1 mM EGTA ⟶ peak B (= 17000 M_r protein).

the possibility of proteolysis during platelet extraction, 10 μg leupeptin, 10 μg pepstatin, 10 μg antipain, and 20 μg TLCK were added per ml of lysis buffer in two different preparations. This resulted in a decrease in the elution rate of the G150 Sephadex column but the protein bound to calmodulin during the affinity chromatography was unchanged. The risk of proteolysis is however not completely excluded and might occur during the concentration steps over sucrose.

3.3. *Effect on the platelet actin-activated Mg^{2+}-dependent ATPase of rabbit skeletal myosin*

In the conditions of our assays the Mg^{2+}-stimulated activity of rabbit skeletal myosin alone was 40 nmol P_i released·min^{-1}·mg $myosin^{-1}$; this value was increased to 300 by platelet actin. Actin alone had no such an ATPase ac-

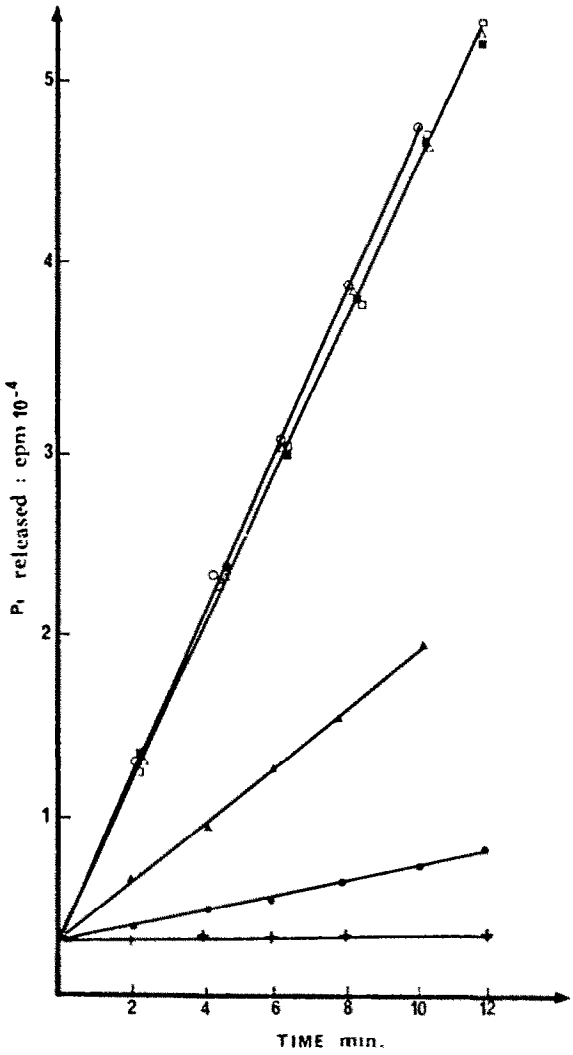

Fig.2. Effect of the 17000 M_r protein on the platelet actin-activated Mg^{2+}-dependent ATPase activity of rabbit skeletal muscle myosin. Conditions are detailed in section 2. Myosin ●; actomyosin ○; actomyosin + calmodulin △; actomyosin + calmodulin + 17000 M_r protein ■: assays performed with and without calcium. Actomyosin + tropomyosin + calmodulin + 17000 M_r protein: assays either in calcium □, or in EGTA ▲. Blank without protein +.

tivity. At 5 mM $MgCl_2$, 2 mM ATP, with or without Ca^{2+}, and under the concentrations of actin and myosin used, neither tropomyosin nor calmodulin had any significant effect on the ATPase. Figure 2 shows that the 17000 M_r protein once associated with calmodulin in a molar ratio of 1:3 with actin did not either affect the activity. However, if the same proportion (1:3) of platelet tropomyosin was included in the assay mixture, the actomyosin ATPase was inhibited by a factor of two in the absence of calcium. Activity was fully restored by 0.2 mM calcium. This leads to a calcium sensitivity** of about 55% (average value obtained with four different preparations of the 17000 M_r protein). These results provide evidence for the presence in pig platelet of an inhibitory factor which shares some properties with troponin I [9].

Since it is well known that calmodulin from various origins have similar properties [21], and since the other proteins have all been isolated from platelets, this thin filament-like complex can be regarded as homogenous. Muszeck et al. [22] and more recently Morimoto et al. [23] reported that calmodulin was the sole calcium sensitizing factor in platelet myosin B. This finding together with the work presented in this paper suggests that the actin-linked regulation of motility in these cells might occur through the calcium sensitive complex of calmodulin with the 17000 M_r protein. The requirement of platelet tropomyosin for the inhibitory activity of the system points out the important regulatory role this protein may play in platelet motility besides its structural function [24].

ACKNOWLEDGEMENTS

This work was supported by grants from the CNRS (ER 060253). E.d.T. wishes to thank Dr A. Weeds for encouragements during the course of this work, the initial phase of which had been undertaken in his laboratory in Cambridge, UK. The authors also acknowledge many stimulating discussions with Drs J. Kendrick-Jones and J.M. Scholey.

REFERENCES

[1] Harris, H.E. (1981) in: Platelets in Biology and Pathology 2 (Gordon, J.L. ed) pp.473–500, Elsevier, Amsterdam.
[2] Kendrick-Jones, J. and Scholey, J.M. (1981) J. Musc. Res. Cell Motility 2, 347–372.
[3] Thomé, F., Huc, C., Coué, M. and Olomucki, A. (1980) J. Musc. Res. Cell Motility 2, 219.
[4] Muszbeck, L. and Harsfalvi, J. (1980) 9th Europ. Congress on Muscle and Motility (Salzburg). Abstract no.62.
[5] Cohen, I. and Cohen, C. (1972) J. Mol. Biol. 68, 383–387.
[6] Côté, G.P. and Smillie, L.B. (1981) J. Biol. Chem. 256, 11004–11010.
[7] Der Terrossian, E., Fuller, S.D., Stewart, M. and Weeds, A.G. (1981) J. Mol. Biol. 153, 147–167.
[8] Der Terrossian, E., Fuller, S.D. and Weeds, A.G. (1979) Hoppe-Seyler's Z. Physiol. Chem. 360, 1376.
[9] Amphlett, G.W., Vanaman, T.C. and Perry, S.V. (1976) FEBS Lett. 72, 163–168.
[10] Côté, G.P. and Smillie, L.B. (1981) J. Biol. Chem. 256, 7257–7261.
[11] Der Terrossian, E., Prulière, G. and d'Albis, A. (1982) Biol. Cell. 45, 251.
[12] Perry, S.V. (1955) Methods Enzymol. 2, 582–588.
[13] Yazawa, M., Sakuma, M. and Yagi, K. (1980) J. Biochem. 87, 1313–1320.
[14] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[15] Laemmli, U.K. (1970) Nature (London) 227, 680–685.
[16] Seals, J.R., McDonald, J.M., Bruns, D. and Jarett, L. (1978) Anal. Biochem. 90, 785–795.
[17] Hartshorne, D.J. and Mueller, H. (1968) Biochem. Biophys. Res. Commun. 31, 647–653.
[18] Greaser, M.L. and Gergely, J. (1971) J. Biol. Chem. 246, 4226–4233.
[19] Castellani, L., Morris, E.P. and O'Brien, E.J. (1980) Biochem. Biophys. Res. Commun. 96, 558–565.
[20] Grand, R.J.A., Perry, S.V. and Weeks, R.A. (1976) in: Excitation Contraction Coupling in Smooth Muscle (Casteels, R. et al. eds) pp.335–341, Elsevier, Amsterdam.
[21] Cheung, W.Y. (1980) Science 207, 19–27.
[22] Muszeck, L., Kuznicki, J., Szabo, T. and Drabikowski, W. (1977) FEBS Lett. 80, 308–312.
[23] Morimoto, K., Kambayaski, J.I., Kosaki, G., Kanda, K., Sobue, K. and Kakiuchi, S. (1982) Biomed. Res. 3, 83–90.
[24] Lazaridès, E. (1976) J. Supramol. Struct. 5, 531(383)–563(415).
[25] Klee, C.B. (1977) Biochemistry 16, 1017–1024.